ACS Medicinal Chemistry Letters
Letter
Cite This: ACS Med. Chem. Lett. 2020, 11, 671−677
pubs.acs.org/acsmedchemlett

Missing Selectivity of Targeted 4β-Phorbol Prodrugs Expected to be Potential Chemotherapeutics

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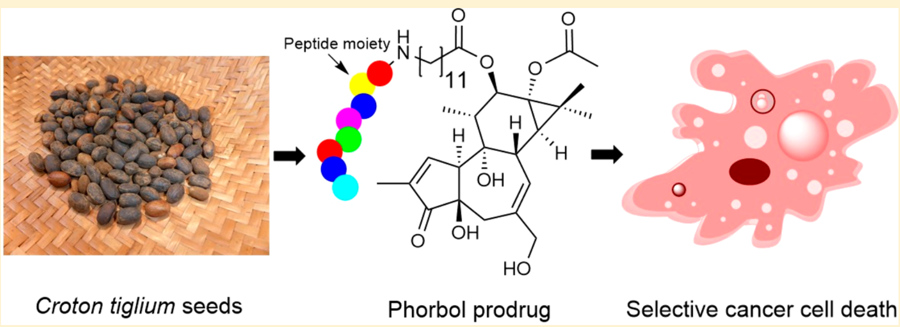
Supporting Information



ABSTRACT: Targeting cytotoxic 4β-phorbol esters toward cancer tissue was attempted by conjugating a 4β-pborbol derivative with substrates for the proteases prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) expressed in cancer tissue. The hydrophilic peptide moiety was hypothesized to prevent penetration of the prodrugs into cells and prevent interaction with PKC. Cleavage of the peptide in cancer tumors was envisioned to release lipophilic cytotoxins, which subsequently penetrate into cancer cells. The 4β-phorbol esters were prepared from 4β-phorbol isolated from *Croton tiglium* seeds, while the peptides were prepared by solid-phase synthesis. Cellular assays revealed activation of PKC by the prodrugs and efficient killing of both peptidase positive as well as peptidase negative cells. Consequently no selectivity for enzyme expressing cells was found.

KEYWORDS: *4β-Phorbol ester, protease-assisted targeting, targeted chemotherapy, prodrug, prostate-specific antigen. prostate-specific membrane antigen*

Prostate cancer (PCa) is a major cause of death by cancer in men in high-income countries.[1] In the initial stage, PCa mainly consists of cells that are androgen-dependent, and the growth can be retarded by hormone therapy.[2] Unfortunately, in later stages hormone refractory cells dominate (castration-resistant prostate cancer, CRPC).[2,3] At this stage the use of common chemotherapeutics is complicated by the slow proliferation of the cancer tissue, since chemotherapeutics like taxanes, doxorubicine, or vincristine target the proliferative stages of cancer. Thus, selectivity is obtained by the faster division rate for cancer cells.[3−6] Therefore, an urgent need for drugs against late-stage PCa exists.

Preclinical evidence supports the idea that drugs targeting protein kinase C (PKC) may be useful in treatment of CRPC.[7] The PKC family comprises ten serine/threonine kinases, which can be divided into three groups: (i) conventional PKC (cPKCs: -α, -βI, -βII, and -γ), (ii) novel PKCs (nPKCs: -δ, -ε, -θ, and -η), and (iii) atypical PKCs (aPKCs: -ζ, -ι, and λ). Expression and function of different PKC isoforms are context-

Special Issue: In Memory of Maurizio Botta: His Vision of Medicinal Chemistry

Received: November 26, 2019
Accepted: December 23, 2019
Published: December 23, 2019

DOI: 10.1021/acsmedchemlett.9b00554
ACS Med. Chem. Lett. 2020, 11, 671−677

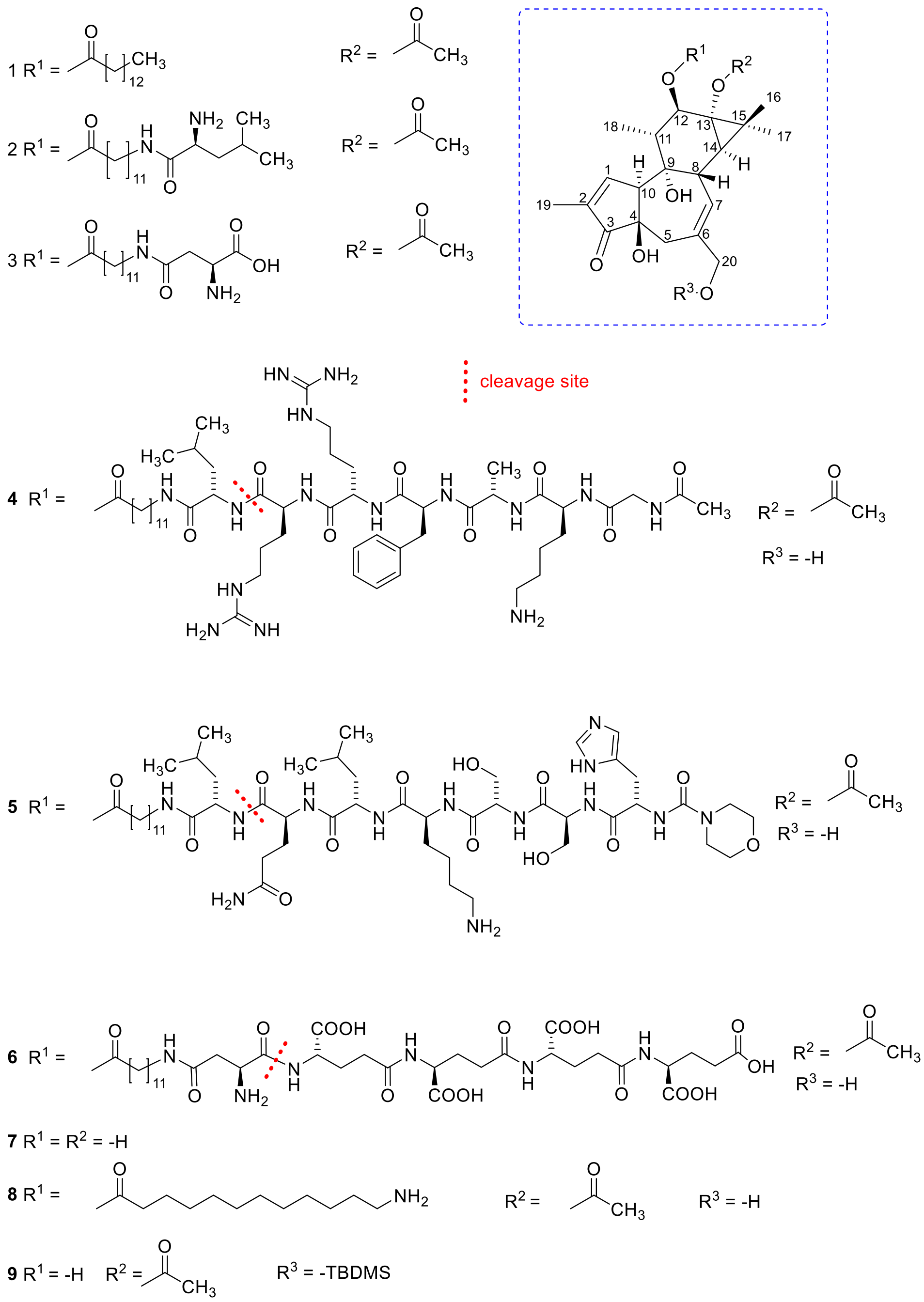


Figure 1. Target compounds and starting material: 4*β*-Phorbol 12-*O*-myristate 13-*O*-acetate (**1**), toxin **2** obtained after cleavage of prodrugs **4** and **5** with hK2 or PSA, respectively, while toxin **3** is obtained after cleavage of prodrug **6** with PSMA. 4*β*-Phorbol (**7**). Compound **8** is the starting material for synthesis of compounds **2**–**3**, and compound **9** for **4**–**6**.

and cell type-specific.[8−11] High expression of PKC*δ* has been reported in prostate cancer, and activation of PKC*δ* induces apoptosis in LNCaP PCa cells.[9,12−15] PKC*ε* is generally overexpressed in PCa and downregulation of PKC*ε* induces apoptosis.[16−20]

PKC-activating diterpenoids related to PMA (**1**, Figure 1) have been in clinical trials.[21−23] Tiglianol tiglate (ECB-46) awaits approval by FDA and EMA for treatment of mast cell tumors in dogs[24] and is entering phase IIA clinical trials for treatment of head and neck squamous cell carcinoma (HNSCC) in humans. Ingenol 3-angelate has under the trade name Picato[R] been approved by the FDA in 2012 as a topical gel for the treatment of actinic keratosis (preliminary stage of skin cancer).[25,26] Since PKC is present in virtually all cells, administration of phorbol esters may affect normal physiology in a broad sense. Selectivity of cancer therapies may be obtained by taking advantage of proteases present in tumors.[27] Prostate specific antigen (PSA), a peptidase expressed by the prostate and PCa, is a diagnostic marker for prostate cancer, and it has been suggested to be involved in cancer invasion and metastasis.[28−32] The missing activity of PSA in the blood caused by complexation with proteins like blood albumin[3,27] makes the enzyme a potential facilitator for selective drug delivery. Since both PSA and prostate specific membrane antigen (PSMA) are expressed by PCa even when they become more undifferentiated and anaplastic, they appear

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ACS Med. Chem. Lett. 2020, 11, 671−677

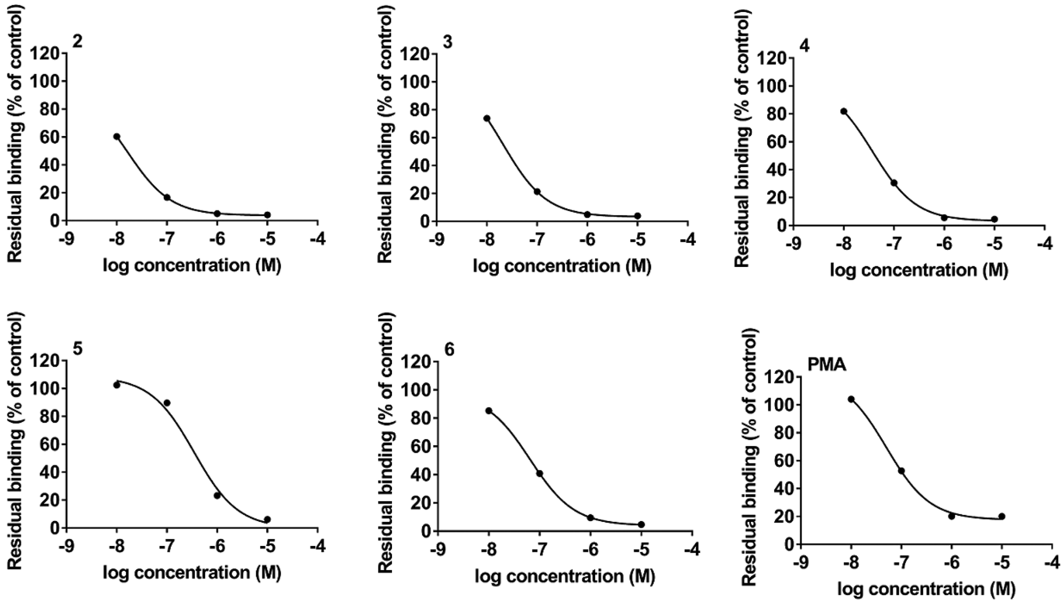

Figure 2. Displacement binding curves of prodrugs **4**−**6**. Toxins **2** and **3** and PMA. Binding of [^{3}H]PDBu (10 nM) to PKCα was measured in the presence of increasing concentrations of the tested compounds. The PKCα was obtained from a lysate if cells overexpressed the enzyme. The data is presented as mean of residual [^{3}H]PDBu binding (% of control) from three parallel samples in a single representative experiment.

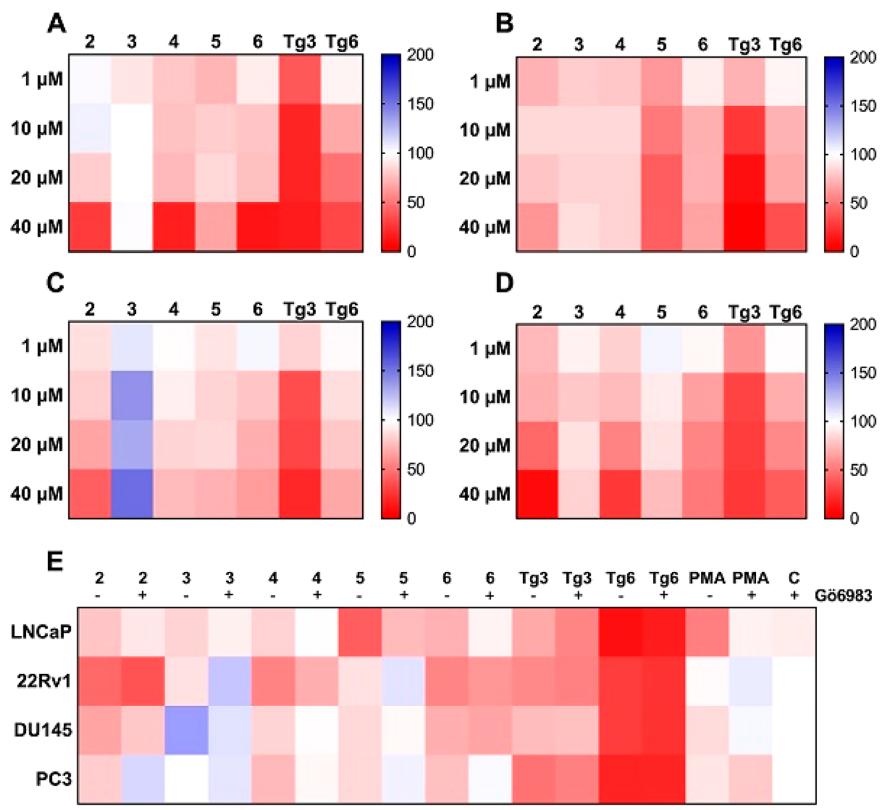

Figure 3. Effects of phorbol prodrugs **4**−**6** and cleavage products **2** and **3** as well as **Tg3** and **Tg6** on viability of PCa cell lines. (A) PC3; (B) LNCaP; (C) DU145; (D) 22Rv1; and the effect of PKC inhibitor Gö6983 (1 μM) on the effect of 20 μM of **2**−**6**, **Tg3**, **Tg6**, and 100 nM PMA on the viability of LNCaP, 22Rv1, DU145, and PC3 cells (E). Cell viability was measured after 72 h incubation with the compounds by utilizing the MTT assay. The data is presented as mean of cell viability (% of control) (n = 3).

to be promising tools in the targeting of toxins for tumors even in CRPC.[33,34] In the case of thapsigargin (**Tg1**, Supporting Information Figure S27) selectivity toward cancer tissue was obtained by conjugation of 8-*O*-12-aminododecanoyl-8-*O*-debutanoylthapsigargin with peptides that are substrates for human glandular kallikrein 2, hK2,[35,36] PSA, or PSMA (mipsagargin, **Tg6**, Figure S27).[37] Mipsagargin has successfully passed clinical trial 2 (For details see Supporting Information paragraph S3).[38] Based on the above findings 4β-phorbol esters **4**−**6** (Figure 1) were designed and expected to display a

DOI: 10.1021/acsmedchemlett.9b00554
ACS Med. Chem. Lett. 2020, 11, 671−677

similar behavior in the organism as the thapsigargin analogs. By conjugating the toxin with a substrate for the proteases, penetration into cells is ideally only possible after enzymatic cleavage by either PSA or PSMA.[30,37] Encouraged by the above-mentioned findings and hypotheses, we have attempted to develop prodrugs of 4β-phorbol esters for selective targeting of PSA- and PSMA-expressing cancer cells.

The starting material 4β-phorbol (**7**) was obtained from seeds of *Croton tiglium* L. (Euphorbiaceae) (for details see Supporting Information paragraph S2.5.1). By a few synthetic steps 4β-pborbol was converted into the cytotoxins **2** and **3** via **8** (Supporting Information S2.5.2). The peptides needed for preparing the prodrugs **4**–**6** were prepared by solid phase syntheses. For syntheses and characterization of the 4β-phorbol toxins and prodrugs see Supporting Information S2.5.3–S2.5.10.

Binding to PKC as Measured by [3H]PDBu Displacement Assay. Compounds **2** and **3** as well as prodrugs **4**–**6**) were tested for binding to the C1 domains of PKCα in a 96-well plate filtration assay as described earlier[39] at a concentration range of 0.01–10 μM. All new compounds (i.e., **2**–**6**) displaced [3H]4β-phorbol 12,13-dibutyrate ([3H]PDBu) as efficiently as PMA (**1**) (Figure 2) except for prodrug **5**, for which an approximately ten times higher concentration was required to achieve a displacement comparable to that of the other compounds. Thus, the presence of a peptide moiety in the prodrugs did not nullify their affinities to the C1 domain of PKCα.

Cell Death as Measured by Cell Viability Assays. The effect of the compounds on the viability of PCa cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 4). The previously established PSMA prodrug mipsagargin (**Tg6**) and the PSMA cleavage product Asp-12-AD-thapsigargin (**Tg3**) were used as reference compounds.[37] The PCa cell lines, used in the present study, represent different types of PCa: androgen-unresponsive DU145 and PC3 cells, which do not express PSA or PSMA, and androgen-responsive 22Rv1 and LNCaP cells, which both are PSA- and PSMA-positive.[40,41] The maximal effect of the PSA cleavage product **2** with the highest concentration gave rise to a reduction in viability of PSA/PSMA-positive LNCaP and 22Rv1 cells to ∼60% and ∼10%, respectively, and in PSA/PSMA-negative DU145 and PC3 cells to ∼40% and ∼30%, respectively (Figure 3). PSA prodrug **5** reduced the viability to below 50% only in LNCaP cells (∼40%) at 20 μM and 40 μM concentrations, whereas PSA prodrug **4** reduced the viability to below 50% at the highest concentration not only in PSA-positive 22Rv1 (to ∼25%) but also in PSA-negative PC3 cells (∼15%) at the highest concentration. To our surprise, the PSMA cleavage product **3** had almost no effect on cell viability in any of the PCa cell lines. The PSMA prodrug **6**, however, decreased the viability to ∼10% at the highest concentration only in the PSMA-negative PC3 cells (Figure 3). The reference compound **Tg3** decreased the viability concentration-dependently in all PC cell lines, and its maximal effect (achieved with the highest concentration) was a reduction in viability to ∼4% for LNCaP, 27% for 22Rv1, 20% for DU145, and ∼15% for PC3 cells. Surprisingly, the other reference compound (i.e., **Tg6**) demonstrated a similar reduction in the viability in PSMA-negative PC3 cells (to ∼32%) as seen for the PSMA-positive LNCaP and 22Rv1 cells (to ∼35% and ∼40%, respectively; Figure 3).

PMA is known to promote PKC-induced apoptosis in the LNCaP cell line.[42] In accordance with this, the pan-PKC inhibitor Gö6983 was able to dampen the effect of 20 μM of compound **5** in LNCaP cells (Figure 3), indicating that the cytotoxic effect indeed is PKC-mediated. The compounds did not induce distinct damage to the cell membranes during the 72-h incubation with any of the concentrations as determined by the LDH test (Figure S2).

Effects on ERK1/2 Phosphorylation and Protein Expression of PKC and PSMA. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) signaling cascade that controls several cellular processes.[43] PKC-mediated ERK1/2 phosphorylation is one of the initial rapid events in PMA-treated LNCaP cells.[42] Since the novel 4β-phorbol-derived compounds compete with PDBu *in vitro*, their ability to modulate ERK activity was investigated in living cells.[44] PSA/PSMA-positive 22Rv1 and PSA/PSMA-negative DU145 PCa cell lines were exposed to 20 μM of compounds **2**–**6**, **Tg3**, and **Tg6** and to 10 nM of PMA for 30 min. Phorbol-derived compounds **2**–**5** induced substantial ERK1/2 phosphorylation in 22Rv1 cells (Figure 4). The ERK1/2 phosphorylation was

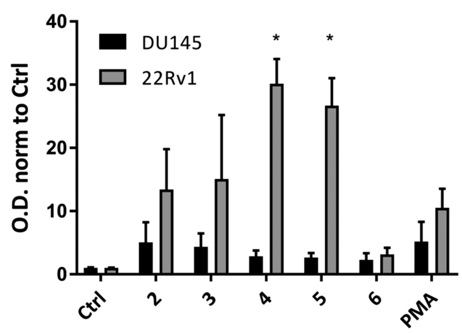

Figure 4. Effects of phorbol derivatives **2**–**6** on ERK1/2 phosphorylation in PC cells. Quantifications from DU145 and 22Rv1 cells. Data is presented as mean + SEM ($N = 3$; $^{*}P < 0.05$ vs ctrl, Welch's *t* test). The cells were treated with 20 μM of different phorbol derivatives and PMA for 30 min. The cells were harvested, and then ERK1/2 phosphorylation was analyzed by using Western blotting with detection as described in Supporting Information S.2.4.4.

even more distinct than after PMA exposure, except in cells treated with compound **6**. The phorbol derivatives also induced ERK1/2 phosphorylation in DU145 cells, but the magnitude of the effect was distinctively smaller than in 22Rv1 cells (Figure 4).

Downregulation of PKC has been suggested to explain the tumor promotion caused by phorbol esters.[10,45] In addition PMA is able to induce androgen receptor downregulation in PCa cells, which is associated with PSMA downregulation.[46,47] To understand the effects of our compounds on the expression of PKC and PSMA, we decided to investigate the effects of the phorbol derivatives on the expression levels of PKCα and PKCδ and PSMA in 22Rv1 cells. A 24 h exposure to 20 μM **3** and **6** increased the expression of PKCα while the incubation with **2** and **4** had no effect. However, PKCα, PKCδ, and PSMA expression was reduced upon 24 h exposure to all compounds, including **3** and **6** (Figure S4 and Table S3). Indeed, our results support the hypothesis that phorbol ester induced down regulation of PKC is associated with down regulation of

DOI: 10.1021/acsmedchemlett.9b00554
ACS Med. Chem. Lett. 2020, 11, 671–677

PSMA. Probably the PKC activating effects of our toxins caused a down regulation of PKC and PSMA.

CONCLUSION

In the present study, we synthesized, characterized, and evaluated the ability of the PSA/PSMA-activable 4β-phorbol ester prodrugs **4−6** and the corresponding cytotoxins **2** and **3** to displace ^{3}H PDBu from PKC and to decrease the viability of PSMA/PSA-positive as well as PSMA/PSA-negative PCa cell lines. In addition, we performed studies on their abilities to increase ERK1/2 phosphorylation. The synthesized PSA/PSMA-activable 4β-phorbol ester prodrugs were designed to contain peptide sequences that are specifically cleaved by either hK2 (i.e., **4**), PSA (i.e., **5**), or PSMA (i.e., **6**). All phorbol-derived compounds showed low nanomolar binding affinity to the C1 domain of recombinant human PKCα, as shown by their displacement of PBDu (Figure 3). Compound **3** induced cytotoxicity only to a limited extent, which may be explained by the lack of ability to penetrate the cell membrane due to its zwitterionic nature at physiological pH. Analogously, compound **3** and prodrug **6** provoked phosphorylation of ERK1/2 only to a limited extent (Figure 3 and 4). Disappointingly prodrugs **4−6** showed no selectivity for PSA/PSMA-positive cell lines (i.e., LNCaP/22Rv1) over PSA/PSMA-negative cell lines (i.e., DU145 and PC3). This observation strongly infers that despite conjugation to a hydrophilic peptide the prodrugs (compounds **4−6**) retain an ability to penetrate cell membranes. The poor activity of **3** and **6** in the viability assay and in the phosphorylation assay indicates that these highly charged molecules can be taken up by the cells only to a limited extent. The results of the present study do not support the hypothesis that the designed PSA/PSMA-targeted prodrugs are capable of providing selective toxicity to PSA/PSMA-expressing PCa cells. Importantly the present results obtained for the known prodrug mipsagargin (**Tg6**, Supporting Information Figure S26) do not support previous observations of selectivity for peptidase expressing cell lines[30] since **Tg6** exhibited clear toxicity both on the PSMA-negative cell line PC3 and on the PSMA-expressing cell lines LNCaP and 22Rv1.[37] A similar poor selectivity has recently been reported by Akinboy et al.[3] for PSA-targeted *O*-8-(morpholine-4-carbonyl-His-Ser-Ser-Lys-Leu-Phe-Gln-Leu-*N*-12-aminododecanoyl)-*O*-8-debutanoyl-thapsigargin (**Tg5**). The missing selectivity of the 4β-phorbol analogs is even more surprising than the missing selectivity of the thapsigargin analogs since the first mentioned are calculated to have lower logP values. In conclusion, the proposed targeted therapy involving conjugation to peptides that are selectively cleaved by proteases present in cancer tissue appears to lack the desired selectivity with 4β-phorbol and thapsigargin analogs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00554.

Experimental procedures, spectral data for all compounds, spectra for all target compounds, and supporting biological data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Ilari Tarvainen and Tomáš Zimmermann have contributed equally to the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This project was funded by the Jane and Aatos Erkko Foundation, Doctoral Programme in Drug Research (University of Helsinki), University of Copenhagen and University of Chemistry and Technology in Prague. We thank Johanna Mosorin for technical assistance.

DEDICATION

This article is written in memory of the valuable contributions professor Maurizio Botta has offered to medicinal chemistry

ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl; CRPC, castration-resistant prostate cancer; 2-CTC, 2-chlorotrityl chloride; 2-Cl-Trt, 2-chlorotrityl; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMAP, 4-(*N,N*-dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide; Fmoc, fluorenylmethyloxycarbonyl; GPCR, G-protein-coupled receptor; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; $InsP_3$, inositol-1,4,5-trisphosphate; MTT, methylthiazolyldiphenyltetrazolium bromide; MW, microwave; NMP, *N*-methyl-2-pyrrolidone; PCa, prostate cancer; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofuran

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DOI: 10.1021/acsmedchemlett.9b00554
ACS Med. Chem. Lett. 2020, 11, 671−677